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TITLE: DNA Replication Arrest and DNA Damage Responses Induced
by Alkylating Minor Groove Binders

PRINCIPAL INVESTIGATOR: Shu-Ru Kuo, Ph.D.
Thomas Melendy, Ph.D.

CONTRACTING ORGANIZATION: Research Foundation of the State
University of New York
Amherst, New York 14228

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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
Alkylating minor groove DNA binder adozelesin is capable of inhibiting DNA replication in treated cells through a trans-acting mechanism. The trans-acting replication factor that becomes deficient upon the action of adozelesin has been identified as replication protein A (RPA). Loss of RPA function can be caused by two possible mechanisms: RPA inactivation and/or loss RPA protein. To study the possibility of RPA inactivation, we purified large amount of RPA from adozelesin-treated cells and examined its function in RPA-dependent assays. We found that RPA purified from cells treated with adozelesin has the same single-stranded DNA binding activity and support nucleotide excision repair as normal RPA, but is not able to support SV40 DNA replication in vitro. Using purified proteins in DNA replication initiation assays, we found that RPA purified from cells treated with adozelesin in not able to be involved in the functional interaction with DNA polymerase α/primase and SV40 large T antigen.

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Introduction:

Adozelesin and bizelesin are analogs of CC-1065, a cyclopropylpyrroloindole (CPI) isolated from Streptomyces zelensis. These CPI drugs are capable of binding to the minor groove of A/T-rich DNA sequences and alkylating the N3 of adenine at 3'-end of the binding sites (2, 22). These two activities contribute to its anti-cancer ability. Binding of CPI does not distort the duplex structure of targeted DNA (2) or cause any DNA strand break (5). Although nucleotide excision repair might be involved in the removal of CPI-induced lesions (6, 8), CC-1065:DNA adducts persist in BSC-1 green monkey cells (23). It is possible that these DNA adducts are been repaired inefficiently in treated cells. At the time this proposal was submitted, both adozelesin and bizelesin showed promising anti-cancer activity in animal studies and entered clinical trials. Unfortunately, adozelesin showed delayed liver toxicity in treated patients and was not recommended for further trials. Bizelesin is still in Phase II clinical trials.

Bizelesin and CC-1065 are capable of binding to both strands of DNA to form interstrand-crosslinks (18), which cause high toxicity in treated cells. Bizelesin treatment does not block DNA replication efficiently but arrest cells in G2/M phase of cell cycle. In contrast, adozelesin inhibits both cellular and viral DNA replication in treated cells with high efficiency through a trans-acting mechanism (11). As shown in my 2nd-year report and in our published results (10), adozelesin selectively induces cellular checkpoint responses in S phase of cell cycle, which depend on the presence of active replication fork progression. Despite the disappointed results from clinical studies, adozelesin is still considered an important agent to study S phase-specific DNA damage responses. The trans-acting replication factor that becomes deficient upon the action of adozelesin has been identified as replication protein A (RPA) (11). RPA is the major eukaryotic single-stranded DNA (ssDNA) binding protein complex initially identified as an essential DNA replication protein (7, 21). This heterotrimeric protein (70, 32 and 14 kDa) also has high affinity for damaged double-stranded DNA (9, 15) and plays critical roles in DNA repair and recombination (7, 21). Interestingly, RPA has also been reported to play a role in regulation of transcription (4, 16, 17). Yeast genetic studies suggest that RPA is involved in the DNA replication checkpoint (13, 19); however, RPA's role in this process is still unclear.

It was first reported a decade ago that extracts from cells treated with UV radiation had a lower activity in cell-free SV40 DNA replication reaction and this reduced activity can be rescued by addition of purified RPA (3). It was since hypothesized that RPA's function in DNA replication is inactivated in cells with damaged DNA. However, in vitro studies from several labs were not able to identify the mechanism that leads to the inactivation of RPA (21). Wang et al. (20) reported that extracts from cells treated with camptothecin had a reduced level of RPA. They suggested that the functional deficiency of RPA in extracts from DNA damaging agents-treated cells was caused by the lower RPA concentration instead of RPA inactivation. However, extracts from adozelesin-treated cells also had a functional deficiency of RPA but without clear change in RPA protein level (11). We therefore decided to purify RPA from adozelesin-treated cells and study its functions in RPA-dependent assays.

Body

During the third year of this Fellowship, I focused on the studies of functional modification of RPA induced by adozelesin treatment. To identify the RPA function in DNA replication that is affected by this modification, I have purified large amount of RPA from either mock- or adozelesin-treated cells and test them in a serial of well-studied RPA-dependent assays.

RPA purification. 16 liters of suspension cultured human 293 cells were either mock-treated or treated with 100 nM adozelesin for 2 hr. Cells were harvested and lysed in hypotonic buffer. Cell extracts were pass through phosphocellulose column. The flow-through that contains RPA was mixed with 20 ml of Q Sepharose and packed into a column. RPA was eluted from Q Sepharose with 250 mM NaCl and applied directly into a 5 ml single-strand DNA cellulose column. 500 mM NaCl was used to wash the column and RPA was eluted with 1.5 M NaCl and 40% ethyleneglycol. RPA was then concentrated with a 1 ml HiTrap Q Sepharose column. RPA purified from mock-treated cells (wt-RPA) using this simplified protocol had >80% purity. However, RPA fraction purified from adozelesin-treated cells (ado-RPA) had only ~30% purity. We used this pair of RPA proteins in RPA-dependent assays (see below).

Since most of the contaminants in ado-RPA fraction are not found in wt-RPA fractions, it is possible that these co-purified proteins might contribute to the activity changes in RPA-dependent assays. Several methods had been tried to further purify RPA but failed to improve the purity. We therefore decided to clone an expressing plasmid for Flag-tagged RPA70 (Fig. 1). This plasmid was then used to transfect 293 cells. Under G418 selection, the Flag-tagged RPA70 consists of about 5% of total cellular RPA70. Immunoprecipitation with anti-Flag antibody showed that this Flag-RPA70 formed complexes with RPA32 and RPA14 in trasfected cells. When treated with DNA damaging agents, Flag-RPA, together with cellular RPA, became extraction resistant and associated with damaged DNA (data not shown). These results suggest that this Flag-RPA is functional in vivo. Adozelesin was then used to treat the transfected 293 cells and Flag-RPA was purified using anti-Flag antibody-conjugated beads. Flag peptides were used to elute Flag-RPA from the beads. In an SDS-PAGE stained with coomassie blue showed that the only polypeptides visible in this fraction are RPA70, RPA32 and RPA14. Since the yield of the Flag-RPA is low, they are only used in key experiments to confirm the results.

RPA-dependent DNA replication and repair assays. Wt-RPA and ado-RPA showed similar affinities to single-stranded DNA in gel mobility shift assays (Fig. 2). When tested in cell-free RPA-dependent SV40 DNA replication reactions, ado-RPA had a much reduced activity (Fig. 3). Interestingly, when tested in cell-free RPA-dependent nucleotide excision repair assays, ado-RPA and wt-RPA had virtually the same activities (Fig. 4). These results suggest that RPA is selectively inactivated as a DNA replication factor in adozelesin-treated cells. Since single-stranded DNA binding activity is not affected, the functional defect of ado-RPA must be contributed by the change in protein-protein interaction. A simplified replication reaction using purified proteins was then used to focus in the potential candidates that involved in the functional change of RPA. Monopolymerase assays were developed at early 90s to study DNA replication initiation. These reactions contain an SV40 origin-containing plasmid for DNA template, purified DNA polymerase α/primase for DNA synthesis, SV40 large T antigen for origin recognition and helicase activity and dNTPs as building blocks. Efficient DNA synthesis in these reactions requires the addition of purified human RPA, which can not be replaced by bacterial single-stranded DNA binding protein SSB. As shown in Fig. 5, ado-RPA can not support DNA synthesis as well as wt-RPA in monopolymerase assays. This data narrows down the RPA functional defect to the three-way interaction among RPA, large T antigen and DNA polymerase α/primase. RPA is known to stimulate DNA synthesis activity of DNA polymerase α using a primed template. As shown in Fig. 6, ado-RPA supports this activity at wt-RPA level, suggests that the functional interaction between ado-RPA and DNA polymerase α is not affected. We are currently testing ado-RPA in large T antigen-dependent DNA unwinding assays and co-immunoprecipitation assays. The preliminary results show that interaction of ado-

RPA to large T antigen is also at wt-RPA level (data not shown). Our results suggest that the functional modification of RPA in cells with damaged DNA is affecting the ability of RPA to be involved in the three-way interaction during the initiation of DNA replication.

Key Research Accomplishments:

Task 1. RPA function in different cell lines. RPA's function in DNA replication is deficient in cells with damaged DNA (11, 12). We have tested RPA activity in several mutation cell lines that are deficient in DNA damage checkpoint functions (12, 14). So far, we are not able to identify the upstream factors that lead to DNA damage-induced RPA inactivation.

Task 2. Cell-free DNA replication assay. We found no evidence that the function of DNA replication factors other than RPA were modulated in cells with DNA damage (11).

Task 3. RPA purification. In the original application, I proposed to purify total RPA from adozelesin-treated suspension-cultured 293 cells. We did purify enough modified RPA to test in several RPA-dependent assays. These results show that RPA purified from adozelesin-treated cells is selectively inactivated as a DNA replication factor (see above). However, the purity of that RPA fraction is only about 30% with other proteins co-purified through several column chromatography. We have therefore generated a plasmid expressing a Flag-tagged RPA70. We have shown that this Flag-RPA70 formed complex with RPA32 and RPA14 and this trimeric Flag-RPA has the same activity as normal RPA in vivo and in vitro (manuscript in preparation). After treated with adozelesin, Flag-RPA can be purified by single step immuno-affinity column chromatography with more than 90% purity. We have been using this purified Flag-RPA to further confirm the functional changes of RPA in cells treated with adozelesin (see Task 4).

Task 4. Characterization of inactive RPA. RPA purified from cells treated with adozelesin retains its single-stranded DNA-binding activity but is selectively inactivated as a DNA replication factor. Its function in nucleotide excision repair (NER) is not affected.

Task 5. Phosphorylation and acetylation of RPA. Our preliminary data suggested that RPA is inactivated through phosphorylation. Treatment of inactivated RPA with phosphatase rescued RPA's activity in DNA replication assays. RPA32 is heavily phosphorylated in cells with DNA damage. However, kinetic assays suggest that RPA32 phosphorylation is not related to RPA inactivation. We are currently focusing on the possibility of RPA inactivation through RPA70 phosphorylation as suggested by studies in yeast cells (1). We can not find RPA been acetylated in cells with damaged DNA.

Task 6. Identification and purification of the trans-acting inhibitor. One of the trans-acting inhibitors was identified as DNA-PK (12).

Task 7. Comparison of trans-acting inhibitors. Bizelesin treatment induces a trans-acting inhibitor that is different from DNA-PK. We have tried to purified this factor through column chromatography. Unfortunately, this bizelesin-induced trans-acting inhibition activity is not very stable and disappeared after two steps of purification.

Task 8. Cell-free DNA repair assay. As described in Task 4, followed a protocol provided by Dr. R. Woods, we have developed a cell-free nucleotide excision repair (NER) assay in the lab.

Reportable Outcomes:

(1) Manuscript:

Liu, J. S.*, **Shu-Ru Kuo***, M. M. McHugh, T. A. Beerman, and T. Melendy (2000). Adozelesin triggers DNA damage response pathways and arrests SV40 DNA replication through replication protein A inactivation. *J. Biol. Chem.* 275:1391-1397.

McHugh, M. M., X. Yin, **Shu-Ru Kuo**, J. S. Liu, T. Melendy, and T. A. Beerman (2001). The cellular response to DNA damage induced by the enediynes C-1027 and neocarzinostatin includes hyperphosphorylation and increased nuclear retention of Replication Protein A (RPA) and trans inhibition of DNA replication. *Biochemistry.* 40:4792-4799.

Liu, J. S.*, **Shu-Ru Kuo***, X. Yin, T. A. Beerman, and T. Melendy (2001). DNA damage by the enediyne C-1027 results in the inhibition of DNA replication by loss of replication protein A function and activation of DNA-dependent protein kinase. *Biochemistry.* 40:14661-14668.

Liu, J.-S.*, **Shu-Ru Kuo***, T. A. Beerman, and T. Melendy (2003). Induction of DNA Damage Responses by Adozelesin Is S Phase-specific and Dependent on Active Replication Forks. *Mol Cancer Ther.* 2:41-47.

Liu, J. S.*, **Shu-Ru Kuo***, T. A. Beerman, and T. Melendy. Intra-nuclear focalization and hyper-phosphorylation of Replication Protein A are mutually independent. *(Submitted).*

Liu, J. S.*, **Shu-Ru Kuo***, and T. Melendy. Replication Protein A (RPA) is selectively inactivated in cell with damaged DNA. *(in preparation).*

Liu, J. S.*, **Shu-Ru Kuo***, and T. Melendy. Cellular responses to stalled replication forks. *(in preparation).*

(*Authors with equal contribution)

(2) Meeting Abstract:

Shu-Ru Kuo, Jen-Sing Liu, Xia Yin, Terry A. Beerman, and Thomas Melendy. Different DNA damaging agents trigger different DNA replication inhibition mechanisms. "Eukaryotic DNA Replication Meeting", The Salk Institute, La Jolla, CA. Sept. 6-10, 2000.

Jen-Sing Liu, Shu-Ru Kuo, Terry A. Beerman, and Thomas Melendy. DNA replication-dependent and -independent mechanisms for DNA damage-induced intra-nuclear redistribution and hyper-phosphorylation of RPA. "Eukaryotic DNA Replication", Cold Spring Harbor Laboratory. Sept. 5-9, 2001.

Jen-Sing Liu, Shu-Ru Kuo, Terry A. Beerman, and Thomas Melendy. Differential cellular responses to stalled DNA replication forks. "Eukaryotic DNA Replication Meeting", The Salk Institute, La Jolla, CA. Aug. 17-21, 2002.

Shu-Ru Kuo, Jen-Sing Liu, Terry A. Beerman, and Thomas Melendy. Anti-cancer agent adozelesin induces RPA focus formation and phosphorylation in S phase cells. "Era of Hope", Orlando, Fl. Sept. 25-28, 2002.

(3) Grant Application:

"Mechanisms of DNA damage triggered S phase checkpoints"
PI: Thomas Melendy (Mentor of this project)
R01 CA89259-01 (NIH/NCI)
Jan, 2001-Dec, 2005

Conclusions:

During the three years of this Fellowship, we have used adozelesin as a DNA damaging agent to study the cellular responses to DNA damage. Our results are summarized as following.

(1) We have demonstrated that adozelesin induces cellular DNA damage checkpoint responses only in S phase cells with active replication fork progression (10). Considering the high growth rate of tumor cells, chemotherapeutic agents that target on S phase of cell cycle certainly will have higher impact on malignant tissues. The delayed liver toxicity of adozelesin may be caused by the inefficient removal of adozelesin:DNA adducts. We believe that members in the family of alkylating minor groove DNA binders have high potential to be efficient anti-tumor agents.

(2) DNA-dependent protein kinase (DNA-PK) was shown to be a trans-acting DNA replication inhibitor (12). The target of DNA-PK that leads to replication arrest is still under investigation.

(3) We have shown that RPA can be used as a marker for S phase DNA damage checkpoint responses (10). Based on the property of RPA, it is possibly that RPA is functioned in vivo as a DNA damage sensor (submitted).

(4) In cells with damaged DNA, RPA is selectively inactivated as a DNA replication factor. The inactivated RPA loss its ability to be involved in the three-way interaction with DNA polymerase α and large T antigen (DNA helicase) to initiate DNA replication.

Based on the finding of this project, we propose to further our research in the following directions:

(1) We are going to identify the mechanism that lead to RPA inactivation. Ore preliminary results suggest that RPA in cells with damaged DNA is inactivated through phosphorylation. The kinase responsible for RPA inactivation will be identified.

(2) Adozelesin is capable of damaging DNA without causing DNA strand breaks. As proposed in our published manuscript (10), adozelesin-induced S phase-specific checkpoint responses are possibly mediated through stalled replication forks. We are going to study the cellular mechanisms induced by replication fork arrest. A better understanding of the molecular interaction on stalled replication forks can be used to design more specific anti-cancer therapeutic agents.

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Figure 1. Oligonucleotides carry the sequences of Kozak signal followed by ATG, nucleotides for Flag epitope (8 amino acid or 24 base) and a BamH 1 restriction enzyme sequence were cloned into pBluescript-SK(+). The coding sequence of RPA70 was then cloned in-frame to the epitope. This Flag-RPA70 was then cloned into pcDNA(3) expression plasmid as shown.

pCMV

Flag

Neo

pcDNA-Flag.RPA70

RPA70
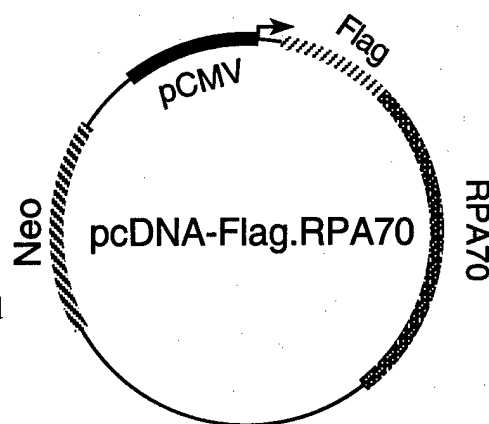
Figure 2. RPA purified from mock-treated cells (wt-RPA) and from adozelesin-treated cells (ado-RPA) were tested in electrophoresis mobility shift assays with a radioactive end-labeled 30-mer single-stranded oligonucleotide.

wt-RPA   ado-RPA

0   12.5 25   50   100 12.5 25   50   100   RPA (ng)

← Probe alone
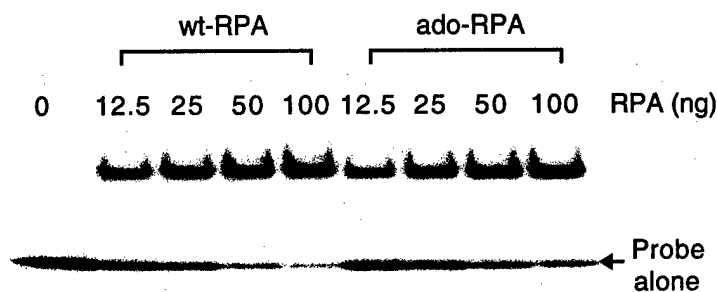
Figure 3. RPA purified from mock-treated cells (wt-RPA) and from adozelesin-treated cells (ado-RPA) were tested in partially reconstituted SV40 origin replication assays. Several components are in these reactions: an SV40 origin-containing plasmids as DNA template, SV40 large T antigen as origin recognition protein and helicase, IIA fraction of 293 cells containing DNA polymerases, purified RFC, PCNA and topoisomerase I together with NTP and dNTP as building blocks. Efficient DNA synthesis in these reactions requires the addition of human RPA protein. Radioactive labeled dATP was also included to label newly synthesized DNA.


Relative Activity vs RPA (ng/µl), wt-RPA and ado-RPA curves.
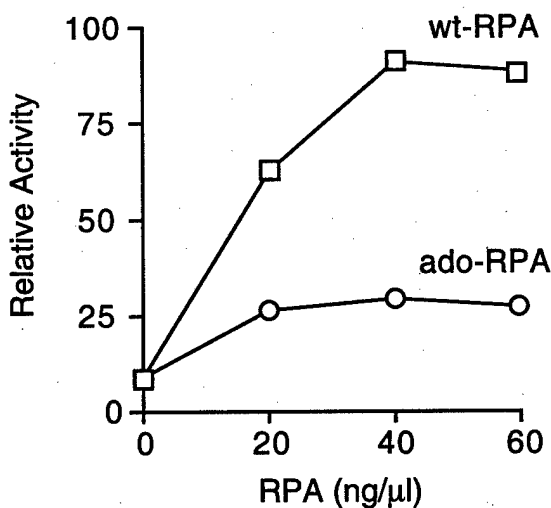
Figure 4.
RPA-dependent nucleotide excision repair (NER)

| | wt-RPA | | | | ado-RPA | | | | |
| 0 | 3.75 | 7.5 | 15 | 30 | 3.75 | 7.5 | 15 | 30 | RPA (ng/µl) |
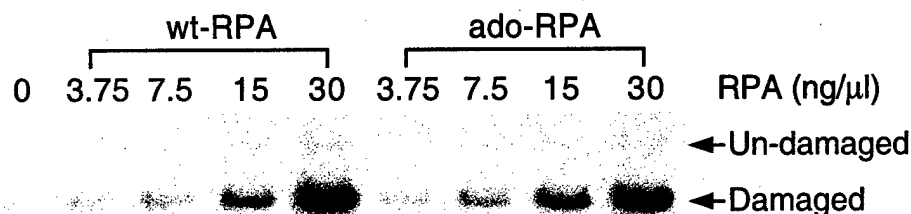
←Un-damaged
←Damaged

assays. Two supercoiled Form I plasmids with different sizes are used in this assay. The un-damaged plasmid is the regular Form I DNA purified through sucrose gradient. The damaged plasmid or NER substrate is purified DNA that is UV-irradiated at 10J/m2, Nth nuclease treated and the Form I DNA purified through sucrose gradient. NER enzymes used in these reactions are partially purified from 293 cell extracts through phosphocellulos column. These reactions require additional purified PCNA and RPA.

Figure 5. RPA purified from mock-treated cells (wt-RPA) and from adozelesin-treated cells (ado-RPA) were tested in monopolymerase assays. The DNA template is an SV40 origin-containing plasmid. Purified SV40 large T antigen is used as origin recognition protein and provide helicase activity to unwind the duplex DNA. RPA is required to stabilize the single-stranded DNA, so DNA polymerase α/primase can synthesize daughter molecules on the template. In this reaction, RPA can not be replaced by either bacterial SSB or yeast RPA.
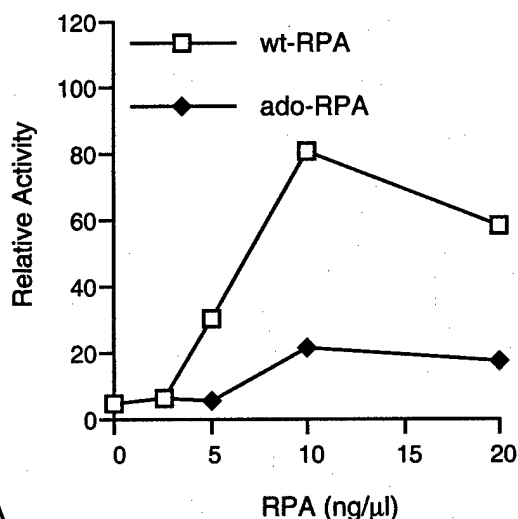
Figure 6. RPA purified from mock-treated cells (wt-RPA) and from adozelesin- treated cells (ado-RPA) were tested in polymerase assays. The DNA template is a long single-stranded DNA with only adenosine (poly dA) annealed with a short oligonucleotide with only thymidine (oligo dT). DNA polymerase α and radioactive labeled dTTP were used to label newly synthesized DNA. While RPA is not essential for this reaction, the presence of RPA greatly stimulate the polymerase activity, which can not be replaced by SSB.
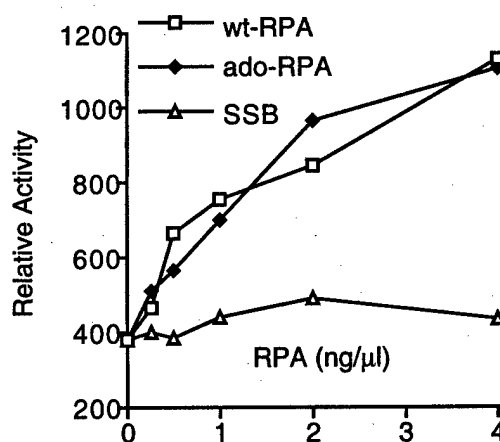
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DNA Damage by the Enediyne C-1027 Results in the Inhibition of DNA Replication by Loss of Replication Protein A Function and Activation of DNA-Dependent Protein Kinase

Jen-Sing Liu, Shu-Ru Kuo, Xia Yin, Terry A. Beerman, and Thomas Melendy

Departments of Microbiology & Biochemistry and Witebsky Center for Microbial Pathogenesis & Immunology, State University of New York at Buffalo, School of Medicine & Biomedical Sciences, Buffalo, New York 14214, and Department of Pharmacology & Therapeutics, Roswell Park Cancer Institute, Buffalo, New York 14263

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DNA Damage by the Enediyne C-1027 Results in the Inhibition of DNA Replication by Loss of Replication Protein A Function and Activation of DNA-Dependent Protein Kinase[†]

Jen-Sing Liu,[‡,§] Shu-Ru Kuo,[‡,§] Xia Yin,[‖] Terry A. Beerman,[‖] and Thomas Melendy*[,‡]

Departments of Microbiology & Biochemistry and Witebsky Center for Microbial Pathogenesis & Immunology, State University of New York at Buffalo, School of Medicine & Biomedical Sciences, Buffalo, New York 14214, and Department of Pharmacology & Therapeutics, Roswell Park Cancer Institute, Buffalo, New York 14263

ABSTRACT: Treatment of cells with the enediyne C-1027 is highly efficient at inducing single- and double-strand DNA breaks. This agent is highly cytotoxic when used at picomolar levels over a period of days. For this study, C-1027 has been used at higher levels for a much shorter time period to look at early cellular responses to DNA strand breaks. Extracts from cells treated with C-1027 for as little as 2 h are deficient in SV40 DNA replication activity. Treatment with low levels of C-1027 (1–3 nM) does not result in the presence of a replication inhibitor in cell extracts, but they are deficient in replication protein A (RPA) function. Extracts from cells treated with high levels of C-1027 (10 nM) do show the presence of a trans-acting inhibitor of DNA replication. The deficiency in RPA in extracts from cells treated with low levels of C-1027 can be fully complemented by the addition of exogenous RPA, and may be due to a C-1027-induced decrease in the extractability of RPA. This decrease in the extractability of RPA correlates with the appearance of many extraction-resistant intranuclear RPA foci. The trans-acting inhibitor of DNA replication induced by treatment of cells with high levels of C-1027 (10 nM) is DNA-dependent protein kinase (DNA-PK). DNA-PK is activated by the presence of DNA fragments induced by C-1027 treatment, and can be abrogated by removal of the DNA fragments. Although it is activated by DNA damage and phosphorylates RPA, DNA-PK is not required for either RPA focalization or loss of RPA replication activity.

When DNA damage occurs during the S phase of the cell cycle, cellular checkpoint pathways are activated to arrest DNA replication and turn on DNA repair mechanisms, or to induce programmed cell death. Although many types of DNA damage are able to physically block replication fork movement, as little as a single damaged site is reported to be able to inhibit all cellular DNA replication (*1*). This indicates that trans-acting mechanisms must be induced to inhibit replication forks distal to the site of damage. Extensive genetic studies have identified multiple genes that are involved in S phase DNA damage checkpoint pathways (*2, 3*). However, how these gene products participate in arresting DNA synthesis requires further study.

The most common agent used to study cellular responses to double-strand DNA breaks (DSBs)[1] is ionizing radiation. However, while gamma irradiation has been shown to induce DSBs, this type of damage makes up only ~10% of the DNA lesions produced, which makes it difficult to separate cellular responses to DSBs from cellular responses to other types of DNA damage. The natural antibiotic enediyne C-1027 is a DNA scission agent that generates both single-strand DNA (ssDNA) breaks and DSBs (*4, 5*). The absence of other types of adducts makes C-1027 a useful reagent for looking at cellular responses to DNA strand breaks.

The well-established in vitro simian virus 40 (SV40) DNA replication system has proven invaluable in investigating DNA damage-induced DNA replication arrest (*6*). Extracts from cells treated with a variety of genotoxic agents have been shown to be deficient in their ability to support SV40 DNA replication in vitro. We have recently shown this to also be true for C-1027 (*7*). However, the mechanisms of this DNA replication inhibition have been shown to be different for different genotoxic agents. Treatment of cells with gamma radiation, camptothecin, or a bifunctional DNA alkylator, bizelesin, induces the presence of a dominant trans-acting inhibitor of DNA replication (*8–11*). Wang et al. (*11*)

* To whom correspondence should be addressed at the Department of Microbiology, 138 Farber Hall, School of Medicine & Biomedical Sciences, SUNY at Buffalo, Buffalo, NY 14214-3000. Tel: (716) 829-3789; Fax: (716) 829-2158; E-mail: tmelendy@buffalo.edu.
[‡] State University of New York at Buffalo.
[§] Authors with equal contribution.
[‖] Roswell Park Cancer Institute.

[1] Abbreviations: RPA, replication protein A; DNA-PK, DNA-dependent protein kinase; DSB, double-strand break; ssDNA, single-strand DNA; SV40, simian virus 40.

reported that the trans-acting inhibitor induced by campto-thecin treatment is DNA-dependent protein kinase (DNA-PK). Their data suggest that inhibition of SV40 DNA replication by DNA-PK may be mediated through phos-phorylation and inactivation of SV40 large T antigen. The identity of the trans-inhibitor induced by bizelesin treatment or gamma radiation remains unknown. However, treatment of cells with two other reagents, UV radiation or the DNA alkylator adozelesin, results in extracts that do not contain a trans inhibitor, but are deficient in replication protein A (RPA) function (*12, 13*). This heterotrimeric (70, 32, and 14 kDa) ssDNA-binding complex is a relatively plentiful nuclear protein that plays important roles in DNA replication, repair, recombination, and transcription [for review, see (*14, 15*)]. It has been shown that during the S phase of the cell cycle, or in response to gamma irradiation, RPA can be detected in focal points within cell nuclei (*16−20*). The 32 kDa subunit of RPA (RPA32) is also hyper-phosphorylated by DNA-PK in response to DNA damage. These modifica-tions appear to have no effect on RPA's DNA replication functions; however, they may be involved in transcriptional activation of DNA repair genes (*14, 21−29*).

We have recently demonstrated that treatment of cells with the DSB agent C-1027 results in a decrease in the ability of extracts to support SV40 DNA replication in vitro. Further, we have shown that such treatment also results in a substantial decrease in the levels of RPA in extracts from treated cells, and a concomitant increase in the levels of RPA in the pellet following extract preparation (*7*). Here we investigate this C-1027-induced redistribution of RPA, and the mechanisms of how DNA replication activity is inhibited in extracts from treated cells.

MATERIALS AND METHODS

Chemicals and Kinase Assays. [α-^{32}P]dATP and [γ-^{32}P]-ATP were obtained from Amersham Pharmacia Biotech. C-1027, a gift from Taiho Pharmaceuticals Co., Ltd., Tokushima, Japan, was diluted in water and stored at −20 °C. DNA-PK activity in cell extracts was evaluated using the SignaTECT DNA-Dependent Protein Kinase System (Promega).

Plasmids and Proteins. The SV40 origin-containing plas-mid pSV011 has been described previously (*30*). SV40 large T antigen (Tag) was purified from recombinant baculovirus-infected High-Five insect cells (Invitrogen) using immuno-affinity chromatography (*31*).

Cell Cultures and Antibodies. Human 293 cells were grown as suspension cultures in S-MEM (Life Technologies) containing 5% (v/v) calf serum. MO59J cells (ATCC) were maintained in DMEM/F12 supplemented with 0.05 mM nonessential amino acids and 10% fetal bovine serum. HeLa cells (ATCC) were maintained in DMEM with 10% fetal bovine serum. Monoclonal antibodies specific to the human RPA 70 and 32 kDa subunits have been described previously (*32*).

Indirect Immunofluorescent Staining. MO59J and HeLa cells grown in two-well chamber slides were treated with 0, 0.1, or 1 nM C-1027 at 37 °C for 2 h. RPA was immuno-stained following the procedure of Swindle et al. (*33*) with minor modifications. Briefly, the cells were washed with PBS with 0.5% Triton X-100, fixed with 3% paraformaldehyde

in PBS, and blocked with 50% normal goat serum in PBS. Monoclonal antibody against RPA32 was used as the primary antibody and incubated overnight at 4 °C. After extensive washing, fluorescein-conjugated goat anti-mouse antibody (Vector Laboratory Inc.) was added as the secondary antibody and incubated at room temperature for 1 h. The cells were washed with 0.5% Triton X-100 in PBS and examined under a Nikon Microphot microscope using a Bio-Rad MRC-1024 confocal imaging system. Adobe Photoshop was used for image processing and printing.

Subcellular Fractionation. The cellular fractionation was a slight modification of the protocol of Zou et al. (*34*). Briefly, 5×10^7 suspension-cultured 293 cells were treated with either 0 or 3 nM C-1027 for 2 h. The cells were harvested and washed with PBS. The cyto/nucleosolic fraction was prepared by extracting protein from the har-vested cells for 10 min on ice using 1 cell pellet volume of 0.5% Triton X-100 in CSK buffer (10 mM PIPES, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl$_2$, 1 mM EGTA). Following centrifugation (10 min at 13000g), the residual nuclear structures were washed 3 times with PBS, and then incubated with 100 μg/mL DNase I in CSK buffer at 37 °C for 15 min. Following digestion, ammonium sulfate was added to 0.25 M, and the suspension was further incubated at RT for another 10 min. The suspension was subjected to centrifugation (10 min at 13000g), and the supernatant was collected and designated the chromatin fraction. The remaining insoluble material was washed with PBS and designated the nuclear matrix. The insoluble nuclear matrix was resuspended in SDS sample buffer with 20 mM Tris-HCl, pH 7.5, 1 M 2-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate, and 10% glycerol.

Cell Extract Preparation and Cell-Free SV40 DNA Replication Assays. Cell extracts were prepared from 293 suspension cells treated with DNA damaging agents at the indicated concentrations for 2 h. In vitro SV40 DNA replication conditions and analyses were described previously (*13*). Quantitation of DNA replication activity was achieved using a Bio-Rad Phosphorimager system to quantitate the [^{32}P]dAMP incorporated in DNA products that ranged from the replication intermediates (RI) to Form I positions on agarose gel analyses. Levels of synthesis are displayed as relative activity, which represents the percent of DNA replication compared to the control reaction for each experi-ment. Control reactions contained 40 μg of control cell extract used in the standard SV40 in vitro replication reaction conditions. Each experiment was carried out between 4 and 8 times; representative results are presented.

Immunoblotting. Each subcellular fraction from mock- or C-1027-treated 293 cells (5×10^6) was mixed with an equal volume of 2× SDS sample buffer (40 mM Tris-HCl, pH 7.5, 4% SDS, 2 M 2-mercaptoethanol). Proteins were then resolved by electrophoresis on 12.5% (w/v) SDS−polyacryl-amide gels and transferred to Hybond-P membrane (Amer-sham Pharmacia Biotech) using NovaBlot (Amersham Phar-macia Biotech) as per the manufacturers' instructions. Membranes were probed with monoclonal antibodies against the two largest subunits of RPA, RPA70 and RPA32. Peroxidase-conjugated goat anti-mouse IgG (Pierce) was used as the secondary antibody and was detected using the Supersignal enhanced chemiluminescent reagent (Pierce) and exposure to X-ray film (Marsh).

Preparation of Protein-Free and DNA-Free Fractions of Cell Extracts. Whole cell extracts from mock- or 10 nM C-1027-treated cells were incubated with 200 ng/μL proteinase K at 37 °C for 30 min, followed by deproteination using phenol/chloroform extraction. The fractions were precipitated with 70% ethanol and 0.3 M sodium acetate, pH 5.0. This nucleic acid fraction was resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA to half the original cell extract volume. In the DNA-PK activation assays, the amount of the nucleic acid fraction added is equivalent to that from the control cell extract used to test for DNA-PK activity.

DNase I (Life Technologies) was immobilized on Affi-gel 10 (Bio-Rad) at 30 units/μL of matrix according to the manufacturers' instructions. After several washes with ethanolamine and PBS, the immobilized DNase I was resuspended in 50 μL of DNase I reaction buffer (20 mM Tris-HCl, pH 8.0, 1 mM ZnCl$_2$, and 1 mM DTT). One microliter of the immobilized enzyme matrix was capable of complete digestion of 500 ng of pUC-19 plasmid at room temperature in less than 20 min. Two hundred microliters of extract from mock- or 10 nM C-1027-treated 293 cells was incubated with 10 μL of immobilized DNase I at room temperature for 20 min. The DNase I matrix was pelleted by centrifugation and removed, and the digested DNA was removed from the extracts by dialysis against Buffer A (Tris-HCl, pH 8.0, and 10 mM 2-mercaptoethanol) with 10% glycerol at 4 °C for 2 h. Control cell extracts without DNase I treatment were dialyzed against the same buffer.

RESULTS

Extracts from C-1027-Treated 293 Cells Show Reduced Levels of in Vitro SV40 DNA Replication. We have previously demonstrated that treatment of human cells with C-1027 results in lower levels of in vitro SV40 DNA replication activity (7). To elucidate the mechanism of this replication arrest, suspension cultured human 293 cells were treated with a wider range of C-1027, and extracts were prepared and tested for their ability to support SV40 DNA replication. As previously shown, compared to mock-treated cell extracts, extracts from C-1027-treated cells are clearly deficient in their ability to support SV40 DNA replication (Figure 1A). This was not due to the presence of drug in the extracts as control experiments showed that addition of up to 20 nM C-1027 to extracts from control cells had little effect on in vitro DNA replication (data not shown) (7).

Reduced DNA replication activity can be due either to the loss of essential DNA replication activities or to the induction of trans-acting inhibitors of DNA replication. These two mechanisms can be differentiated by using cell extract mixing experiments to see if extracts from treated cells are capable of inhibiting the replication activity of control cell extracts (8–10, 13). Similar to previously published results, extracts from cells treated with low levels (1 or 3 nM) of C-1027 do not contain a trans-acting inhibitor (Figure 1B), consistent with the loss of one or more activities essential for DNA replication (7). However, when cells were treated with higher levels of C-1027 (10 nM), a potent trans-acting DNA replication inhibitor was induced (Figure 1B, filled squares). These results indicate that the cellular concentration response curve to treatment with C-1027 is biphasic, an observation not previously reported. Upon treatment of cells
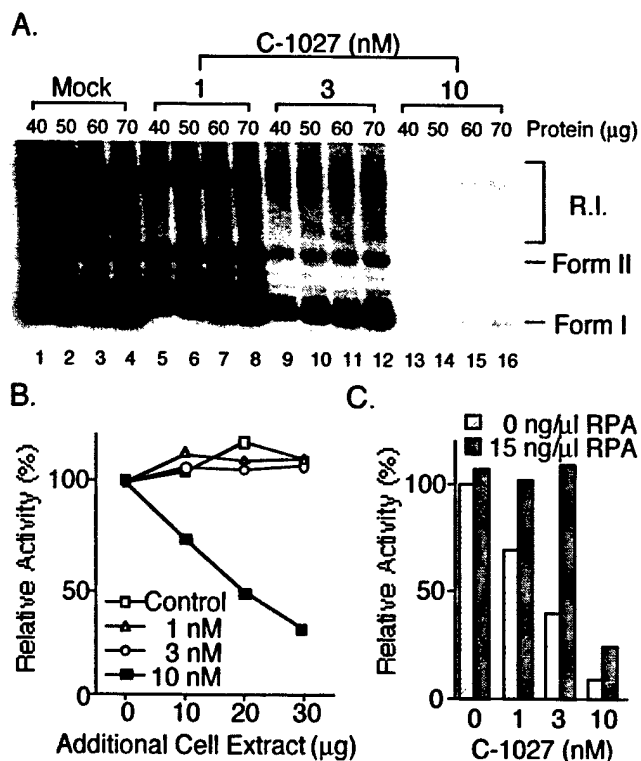
Figure 1: Treatment of cells with C-1027 results in inhibition of in vitro SV40 DNA replication through loss of RPA activity and, at high levels of C-1027, induction of a trans-acting inhibitor. (A) 40—70 μg of extract from cells treated with 0, 1, 3, or 10 nM C-1027 for 2 h was used in in vitro SV40 DNA replication assays. The migration of supercoiled (Form I) DNA, open circular (Form II) DNA, and θ form replication intermediates (R.I.) is indicated to the right of the panel. (B) 0—30 μg of extract from cells treated with 0 (open squares), 1 (triangles), 3 (circles), or 10 nM (filled squares) C-1027 was mixed with 40 μg of mock-treated cell extract and tested in SV40 DNA replication assays. In 6 independent experiments, no trans inhibition was detected in extracts from 1 or 3 nM treated cells. Levels of trans inhibition by extracts from cells treated with 10 nM C-1027 were similar in all experiments. (C) 0 or 15 ng/μL RPA was added to SV40 DNA replication reactions with extracts from cells treated with 0—10 nM C-1027. Four independent experiments showed the addition of RPA to extracts from 1 or 3 nM C-1027-treated cells rescued DNA replication levels to 100 ± 5% of the control. Rescue of DNA replication in extracts from cells treated with 10 nM C-1027 by addition of RPA was always <25% of control reactions. Relative activity (%) indicates the percent of DNA synthesis seen in comparison to levels of synthesis seen with the control reaction, 40 μg of extract from mock-treated cells with no additional RPA.

with low levels of drug, one or more essential DNA replication activities are lost, while at high levels of drug a trans-acting DNA replication inhibitor is induced.

Reduced SV40 DNA Replication in Extracts from Cells Treated with Low Doses of C-1027 Is Mediated through Loss of RPA Function. It has been shown that for some types of DNA damage, addition of exogenous replication protein A (RPA) is capable of rescuing the DNA replication activity of extracts from treated cells (12, 13), while for other types of DNA damage it cannot (8–11). We therefore tested whether the inhibition of DNA replication upon treatment of cells with low doses of C-1027 is due to a loss of RPA function. Addition of purified RPA (to 15 ng/μL) was sufficient to fully rescue SV40 DNA replication activity in extracts from cells treated with either 1 nM or 3 nM C-1027 (Figure 1C). Addition of RPA to mock-treated extracts
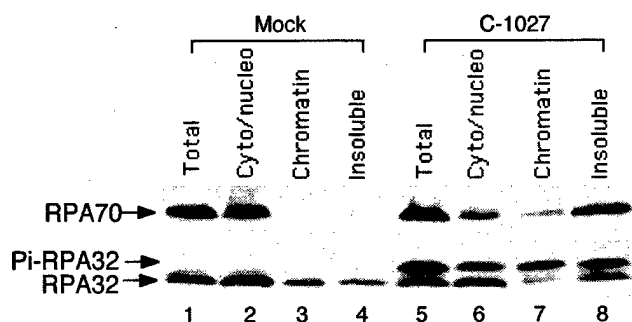
FIGURE 2: A large fraction of the cellular RPA becomes stably associated with the nuclear matrix following treatment with C-1027. Subcellular fractions from mock- and C-1027-treated 293 cells (5 × 10^5) were analyzed via immunoblotting with RPA70 and RPA32 monoclonal antibodies. The amount of each fraction loaded onto the gel represents the following percentage of the total volume of that fraction: Total (lanes 1 and 5), 0.8%; Cyto/nucleo (lanes 2 and 6), 1%; Chromatin (lanes 3 and 7), 3%; Insoluble (nuclear matrix) (lanes 4 and 8), 3%.

FIGURE 3: C-1027 treatment induces rapid, DNA-PK-independent, focalization of RPA. Monolayer cultured HeLa cells (A−C) or DNA-PK$^{(-/-)}$ (MO59J) cells (D−F) were grown on coverslips and treated with 0 (A and D), 0.1 (B and E), or 1 nM (C and F) C-1027 for 2 h. The cells were then washed with 0.5% Triton X-100 to permeabilize the cell and nuclear membranes and remove the free nucleosolic RPA. The slides were then treated with paraformaldehyde to fix the remaining proteins, and then stained for RPA with monoclonal antibody against RPA32 and fluorescein-labeled goat anti-mouse antibody. The nuclei were visualized using a Bio-Rad MRC-1024 confocal microscopic imaging system.

showed little effect on DNA replication activity (tested up to 30 ng/μL; Figure 1C and data not shown). Since the role of RPA in DNA replication is unique, this indicates that the primary mechanism of inhibition of DNA replication following treatment of cells with low doses of C-1027 is through loss of RPA function. Since RPA has never been shown to be able to compensate for deficiency in any other DNA replication protein (*14, 15*), this also demonstrates that all the other essential factors in these extracts remain fully functional for in vitro DNA replication. Conversely, addition of RPA was not sufficient to rescue SV40 DNA replication activity in extracts from 10 nM C-1027-treated cells (Figure 1C). This is consistent with the cell extract mixing experiments showing that a trans inhibitor is induced upon treatment with 10 nM C-1027 (Figure 1B).

Intranuclear Redistribution of RPA. We have previously shown that RPA becomes resistant to extraction by hypotonic lysis in cells treated with C-1027 (*7*). The fact that more than 60% of the total RPA becomes extraction-resistant (Figure 2) (*7*) suggested that this could be the cause of the loss of RPA function in in vitro SV40 DNA replication assays (Figure 1C). To investigate whether RPA is being targeted to the chromatin or to the nuclear matrix, 293 cells were mock-treated or treated with low levels of C-1027 and then separated into three fractions: a combined cytosolic and nucleoplasmic fraction (prepared by treatment of cells with nonionic detergent under low ionic strength to solubilize the cell and nuclear membranes), a chromatin-bound fraction (prepared by extensive DNase digestion of the remaining pellet followed by high-salt extraction), and an insoluble nuclear matrix fraction (see Materials and Methods for details). These fractions were monitored for RPA levels using immunoblotting (Figure 2). For mock-treated cells, the majority of RPA (70−80%) was found in the cyto/ nucleosolic extract (lanes 1 and 2), while very little RPA was found in the chromatin-bound or insoluble nuclear matrix fractions (lanes 3 and 4), consistent with previously published results (*35*). However, cyto/nucleosolic extracts from cells treated with low levels of C-1027 showed a substantial reduction in levels of the RPA complex (as evaluated by levels of RPA70, lanes 5 and 6). Following drug treatment, RPA levels were increased in both the chromatin-bound fraction (lane 7) and to an even greater degree in the insoluble
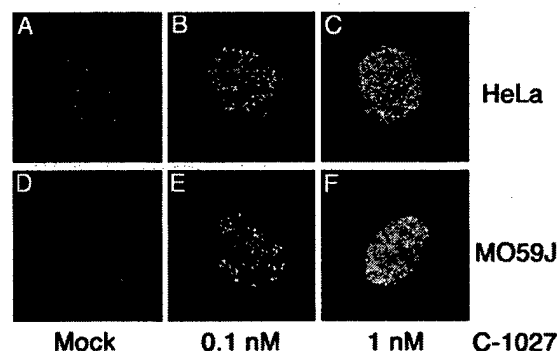
nuclear matrix fraction (lane 8). In C-1027-treated cells, the chromatin-bound RPA population showed a much higher percentage of hyper-phosphorylated RPA32 than the other fractions (lane 7).

To further understand this RPA redistribution, indirect immunostaining was used to monitor the extraction-resistant RPA in drug-treated cells. HeLa cells were treated with low levels of C-1027 for 2 h. The cells were then washed with nonionic detergent (using the same buffer conditions as in Figure 2) before fixation (see Materials and Methods). This nonionic detergent wash results in the solubilization of both the cell and nuclear membranes, thereby washing away both cytoplasmic and nucleoplasmic proteins. This treatment has been shown to remove the vast majority of the RPA from untreated cells, which is present in the loosely bound nucleosolic fraction (*20*). The extraction-resistant RPA was then visualized using a monoclonal antibody against RPA32 and fluorescein-conjugated secondary antibody. Less than half (∼40%) of mock-treated cells showed very low levels of RPA present as small faint nuclear foci (Figure 3A), consistent with previous studies showing RPA's presence at replication foci (*16−20*). Nuclei from the remaining mock-treated cells showed no detectable staining of RPA higher than background. However, cells treated with C-1027 showed strong staining of intranuclear RPA foci in more than 90% of treated cells (Figure 3B). Furthermore, the intensity of the RPA signal and the number of foci increased with increasing levels of drug (Figure 3B,C).

DNA-dependent protein kinase (DNA-PK) is the primary kinase responsible for DNA damage-dependent hyper-phosphorylation of RPA32 (*14*). Therefore, DNA-PK$^{(-/-)}$ MO59J cells were used to test whether RPA focus formation is dependent on DNA-PK. As shown in Figure 3, RPA focalization appears to be unaffected by the absence of DNA-PK in these cells (compare panels A−C to panels D−F). Therefore, we conclude that RPA focalization is not dependent on RPA32 hyper-phosphorylation by DNA-PK.

Extracts from Cells Treated with High Levels of C-1027 Show Induction of DNA-PK Activity. Since treatment of 293 cells with high levels of C-1027 induces the presence of a
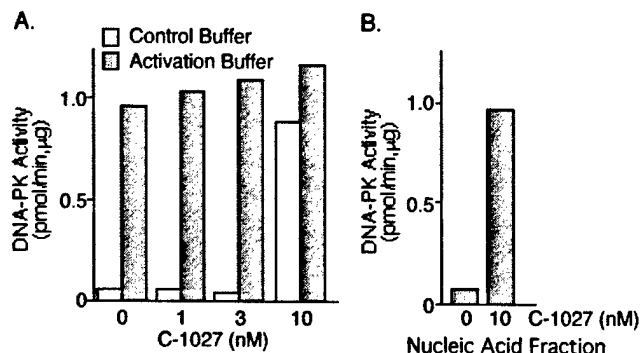
FIGURE 4: Treatment of cells with high levels of C-1027 generates DNA fragments sufficient to induce DNA-PK activity. (A) 40 μg of total protein from hypotonic extracts of 293 cells treated with the indicated concentrations of C-1027 for 2 h (prepared as in Figure 1) was assayed for DNA-PK activity. Control buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) assays show the level of preactivated DNA-PK kinase activity. Activation buffer is the same as control buffer but also contains 100 ng/μL sheared calf thymus DNA to activate any additional DNA-PK in the extracts. In three independent experiments, DNA-PK activity levels by extracts from cells treated with 0, 1, or 3 nM C-1027 ranged from 0.05 to 0.1 pmol min^{-1} μg^{-1}, while activity in extracts from cells treated with 10 nM C-1027 ranged from 0.8 to 1.0 pmol min^{-1} μg^{-1}. Addition of activation buffer to any cell extract resulted in DNA-PK activity levels ranging from 0.9 to 1.2 pmol min^{-1} μg^{-1}. Assays are capable of measuring from 0.02 to 2.0 pmol min^{-1} μg^{-1}. (B) Nucleic acid fractions were prepared from 40 μg of extract from the cells treated with either 0 or 10 nM C-1027 (as in panel A, 0 and 10 nM, open bars) by protein digestion, extraction, and ethanol precipitation of the nucleic acids. These nucleic acid fractions were each added to 40 μg of control extract (as in panel A, 0 nM, open bar), which was then assayed for DNA-PK activity. In three independent experiments, addition of the nucleic acid fraction from control cells to control cell extract resulted in <0.15 pmol min^{-1} μg^{-1} of DNA-PK activity. Addition of nucleic acid fraction from cells treated with 10 nM C-1027 to control cell extracts resulted in DNA-PK activity ranging from 0.9 to 1.1 pmol min^{-1} μg^{-1}.

trans-acting inhibitor of SV40 DNA replication (Figure 1B), and elevated DNA-PK activity has been reported to act as an inhibitor of in vitro SV40 DNA replication (*11, 24*), we examined DNA-PK activity in extracts from cells treated with C-1027. DNA-PK activity levels in extracts from cells treated with 1 and 3 nM C-1027 were indistinguishable from DNA-PK activity levels in control cell extracts (Figure 4A, open bars), indicating that DNA-PK activity is not induced in these extracts. However, extracts from cells treated with 10 nM C-1027 showed a dramatic increase in DNA-PK activity, almost to levels seen with addition of DNA-PK activation buffer to any of the extracts (Figure 4A, compare open bars at 10 nM to striped bars). DNA-PK activation buffer contains fragmented calf thymus DNA sufficient to activate endogenous DNA-PK present in tested extracts (Figure 4A, compare striped bar with open bar for DNA-PK assay results with extracts from '0 nM' C-1027-treated cells). The fact that DNA-PK activity is induced only upon treatment with 10 nM C-1027 is consistent with the possibility of DNA-PK activity being the trans-acting replication inhibitor induced in extracts from cells treated with high levels of C-1027 (Figure 4A and Figure 1B).

The similarity in the DNA-PK activity levels in extracts from cells treated with high levels of C-1027 and in control extracts with the addition of exogenous DNA fragments (present in the DNA-PK activation buffer) suggested that the activation of DNA-PK in extracts from drug-treated cells

might be due to the presence of DNA fragments in these extracts. Nucleic acid fractions were prepared from mock- and C-1027-treated cell extracts and compared for their ability to activate the DNA-PK present in control cell extracts. The addition of the nucleic acid fraction from mock-treated cell extracts to extracts from mock-treated cells did not result in an appreciable increase in DNA-PK activity (Figure 4, compare the striped bar labeled '0 nM C-1027' in panel B to the open bar of '0 nM C-1027' in panel A). However, the addition of the nucleic acid fraction from extracts from cells treated with high levels of C-1027 (10 nM) to extracts from mock-treated cells resulted in a dramatic increase in DNA-PK activity (Figure 4, compare '10 nM C-1027' bar in panel B to the striped bars in panel A). These results clearly indicate that treatment of cells with 10 nM C-1027 causes enough DSBs to release sufficient DNA fragments to fully activate the DNA-PK present in hypotonically prepared cell extracts.

DNA Fragment-Induced DNA-PK Activity Is the Trans-Acting Inhibitor in Extracts from Cells Treated with High Levels of C-1027. To determine whether the C-1027-induced DNA fragments were responsible for the trans-acting DNA replication inhibitory activity in extracts from cells treated with high levels of C-1027, the DNA fragments were removed from these extracts. Immobilized DNase I was used to exhaustively digest any DNA present in extracts from 10 nM C-1027-treated cells, and the remaining small fragments were removed by dialysis (see Materials and Methods). DNA-PK assays were used to demonstrate that after this treatment, DNA-PK activity was reduced to levels similar to those seen in mock-treated cell extracts (data not shown). Cell extract mixing experiments were then used to test for the presence of trans-inhibitor activity as in Figure 1B. Before DNase I treatment, addition of extract from cells treated with high levels of C-1027 showed a strong inhibition of DNA replication by control cell extracts (Figure 5A, filled triangles; and Figure 1B, filled squares). After DNase I treatment of the extract, trans inhibition was no longer seen (Figure 5A, open triangles). DNase I treatment had little effect on mock-treated cell extracts (Figure 5A, open circles and squares). These results indicate that the trans inhibitor induced in extracts from cells treated with 10 nM C-1027 is dependent upon DNA fragments in these extracts.
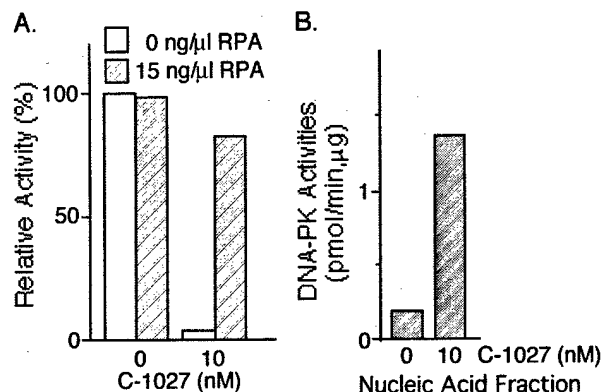
DNase I-treated extracts from cells treated with high levels of C-1027 also did not show the ability to appreciably stimulate the basal level of DNA replication (Figure 5A, compare open triangles to open squares and circles). This result could be explained if these extracts were also deficient in limiting DNA replication activity. Indeed, the DNase I-treated extracts from cells treated with high levels of C-1027 were not capable of supporting appreciable levels of SV40 DNA replication on their own (Figure 5B, striped bars). Since RPA activity is deficient in extracts from cells treated with low levels of C-1027 (Figure 1C), we anticipated that this would also be true for extracts from cells treated with high levels of C-1027. RPA was added back to the DNase I-treated extracts from cells treated with high levels of C-1027 to see if RPA could complement these extracts for in vitro SV40 DNA replication activity. The addition of 15 ng/μL RPA to DNase I-treated extracts from cells treated with high levels of C-1027 was able to rescue SV40 DNA replication to levels consistent with control cell extracts
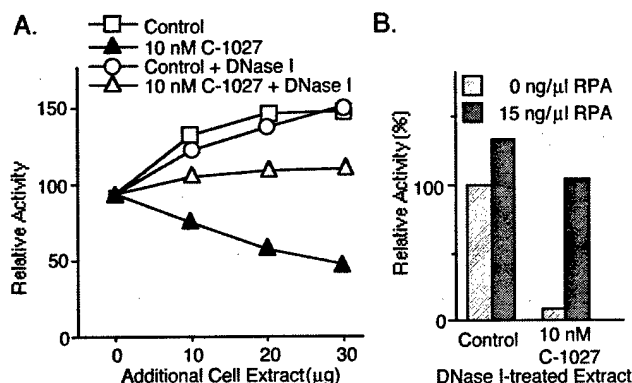
FIGURE 5: DNase I treatment eliminates the trans inhibitor in extracts from cells treated with high levels of C-1027. (A) 40 μg of extract from mock-treated cells was mixed with 0–30 μg of DNase I-treated extract from mock- or 10 nM C-1027-treated cells. These mixtures were then tested for the ability to support in vitro DNA replication. In three independent experiments, the addition of 30 μg of extract from cells treated with 10 nM C-1027 resulted in >40% inhibition of DNA replication by 40 μg of control cell extract. The addition of 30 μg of the same extract first treated with DNase I to 40 μg of control cell extract always showed DNA synthesis levels greater than that seen with 40 μg of control cell extract alone. (B) 0 or 15 ng/μL purified RPA was added to SV40 DNA replication assays carried out with 40 μg of DNase I-treated extracts from either mock- or 10 nM C-1027-treated cells. In three independent experiments, the level of DNA replication by 40 μg of extract from cells treated with 10 nM C-1027 was always < 10% of levels seen with 40 μg of control cell extract. Addition of 15 ng/μL RPA to 40 μg of extract from cells treated with 10 nM C-1027 resulted in DNA replication levels of 90–110% of that seen with 40 μg of control cell extract. Relative activity (%) indicates the percent of DNA synthesis seen in comparison to levels of synthesis seen with the control reaction, 40 μg of untreated extract from mock-treated cells with no additional RPA.

FIGURE 6: SV40 DNA replication in extracts of C-1027-treated DNA-PK$^{(-/-)}$ cells. (A) SV40 DNA replication was carried out with extracts from 0 or 10 nM C-1027-treated MO59J (DNA-PK$^{(-/-)}$) cells in either the presence (open bars) or the absence (striped bars) of 15 ng/μL exogenously added RPA. In three independent experiments, the levels of DNA replication by 40 μg of extract from MO59J cells treated with 10 nM C-1027 resulted in <5% of the levels of DNA synthesis seen with 40 μg of control extract from MO59J cells. Addition of 15 ng/μL RPA to the extracts from the C-1027-treated cells resulted in levels of DNA synthesis ranging from 77 to 83% of that seen with the control cell extract alone. Relative activity (%) indicates the percent of DNA synthesis seen in comparison to levels of synthesis seen with the control reaction, 40 μg of extract from untreated MO59J cells with no additional RPA. (B) Nucleic acid fractions from 40 μg of extract from 0 or 10 nM C-1027-treated MO59J cells were added to 40 μg of extract from mock-treated 293 cells and assayed for DNA-PK activity. In three independent experiments, addition of the nucleic acid fraction from 40 μg of extract from untreated MO59J cells to 40 μg of untreated 293 cell extract resulted in 0.1–0.2 pmol min^{-1} μg^{-1} of DNA-PK activity. Addition of the nucleic acid fraction from 40 μg of extract from MO59J cells treated with 10 nM C-1027 resulted in DNA-PK activity ranging from 1.2 to 1.4 pmol min^{-1} μg^{-1}.

(Figure 5B, compare 10 nM solid bar to control bars). These results are consistent with those in Figure 1C, and show that once the trans-acting inhibitory activity is removed from extracts of cells treated with high levels of C-1027, the only other mechanism of SV40 DNA replication inhibition is through loss of RPA function.

It has been reported that DNA fragments are capable of competing for essential DNA replication factors and can thus inhibit in vitro SV40 DNA replication (36). To verify that the trans inhibitor of DNA replication is activated DNA-PK, and not the DNA fragments themselves, experiments similar to those described above were performed using DNA-PK$^{(-/-)}$ MO59J cells. MO59J cells were treated with 0 or 10 nM C-1027 as above and tested for the presence of a trans-acting DNA replication inhibitor in the cell extracts. Using cell extract mixing experiments, no inhibitor was detected in extracts from MO59J cells treated with 10 nM C-1027 (data not shown). Furthermore, the addition of RPA alone to these extracts was capable of rescuing SV40 DNA replication activity (Figure 6A). As a control to demonstrate that C-1027 is capable of inducing comparable fragmentation of DNA in MO59J cells, nucleic acid fractions were prepared from extracts of MO59J cells treated with 10 nM C-1027 and used to stimulate DNA-PK activity in control 293 cell extracts (as was done in Figure 4B). Figure 6B clearly demonstrates that treatment of MO59J cells with 10 nM C-1027 does induce the presence of DNA fragments in cell extracts, sufficient to activate DNA-PK in control cell extracts. Taken together, these results indicate that the

activation of DNA-PK, not the DNA fragments themselves, is responsible for the trans-acting inhibition of SV40 DNA replication seen in extracts from 293 cells treated with high levels (10 nM) of C-1027.

DISCUSSION

We have shown that treatment of cells with the DNA strand break agent, C-1027, causes both loss of RPA replication activity and induction of DNA-PK activity. Both of these effects have been demonstrated previously in cells treated with different DNA damaging agents. However, this is the first report showing that both mechanisms can be induced by a single DNA damaging agent in a dose-dependent manner. This is further evidence that there appear to be multiple S phase DNA damage checkpoint pathways to turn off DNA synthesis; and that these pathways can be activated differentially, in response both to different types of DNA lesions as well as to the amount of DNA damage.

We have demonstrated that RPA becomes extraction-resistant (associating with the nuclear matrix fraction) and forms foci in cells treated with C-1027 (Figures 2 and 3). These foci arise rapidly following DNA damage and are independent of DNA-PK (Figure 3). Other proteins have also been shown to form intranuclear foci in response to treatment of cells with genotoxic agents. Mre11, 53BP1, and histone H2AX are reported to form nuclear foci at DNA DSBs within a short time after induction of damage (37–39). RPA has been shown to form foci in response to ionizing radiation,

and is believed to bind to these DSBs through subsequent nuclease action on the DSBs, which creates areas of ssDNA. RPA is also capable of binding damaged dsDNA in the absence of strand breaks (*40, 41*). Since RPA binds to both ssDNA and damaged dsDNA and is involved in several DNA repair pathways, one would expect that RPA would form a wide variety of DNA damage-induced foci, only a subset of which would co-localize with these other markers. This suggests that RPA could be a more universal DNA damage marker than other proteins that focalize in response to DNA damage. These questions are currently under investigation.

Another question remaining is whether the transfer of RPA to DNA damage-induced intranuclear foci is responsible for the lack of sufficient RPA to support DNA replication. When cells are treated with 3 nM C-1027, it is estimated that ~60% of the total RPA is transferred to the extraction-resistant fraction, resulting in a concomitant decrease in the amount of RPA in the hypotonic cell lysates (Figure 2). A decrease of 60% of the RPA in these extracts would result in a substantial decrease in DNA replication activity. It has also been suggested that RPA might be inactivated in UV- or adozelesin-treated cells (*12, 13, 36*). If this is the case, loss of RPA function upon DNA damage could be due to more than one mechanism. The biochemical function of RPA purified from cells subjected to DNA damage is currently under investigation.

The available biochemical and cell staining data suggest a unique role for RPA in cellular responses to DNA damage. Extrapolating to the cellular level, we hypothesize that there is a large pool of RPA in normal cell nuclei that is available for DNA replication, recombination, or repair. Once the appropriate signals are triggered, this pool of available RPA is rapidly targeted to sites of DNA replication (at replication foci once S phase begins) or to DNA repair complexes (at sites of damaged DNA, which initiate foci when cells are subjected to DNA damage). The amount of RPA recruited to DNA replication foci is a small proportion of the intranuclear RPA population (*20*), suggesting that during S phase the majority of RPA is still available to form DNA damage-induced foci. However, DNA damage results in a much more substantial depletion of the pool of available RPA, which likely helps inhibit DNA replication, thereby assisting in S phase DNA damage checkpoint responses.

Whether DNA-PK is directly involved in cellular DNA damage-induced replication arrest remains unclear. We have demonstrated in this study that the induction of DNA-PK activity, and the resultant inhibition of SV40 DNA replication in extracts from cells treated with high levels of C-1027, can be directly attributed to the presence of DNA fragments in extracts from treated cells (Figures 4 and 5). And it has been shown that activated DNA-PK is capable of inhibiting SV40 DNA replication in cell-free assays (*11, 24*) (Figures 4 and 5). However, it has been suggested by Wang et al. (*11*) that the inhibition of SV40 DNA replication by DNA-PK may act through phosphorylation and inactivation of the viral replication protein, SV40 large T antigen. This preliminary result needs to be verified; further, it is unknown whether there may be a cellular replication protein whose function is also targeted by DNA-PK. It is currently believed that DNA-PK does not play a primary role in DNA damage checkpoint control (*42, 43*). However, it is possible that DNA-PK activation may play a secondary checkpoint role

at higher levels of DNA damage. In addition, ATM and ATR kinases, both close relatives of DNA-PK, are directly involved in primary DNA damage checkpoint responses (*43*), and ATM, like DNA-PK, has been shown to be activated in response to DNA strand breaks (*44*). Therefore, whether or not DNA-PK is directly involved in the initial inhibition of cellular DNA replication in response to DNA damage, it may be involved in a secondary response at higher levels of DNA damage. Further, DNA-PK's effect on SV40 DNA replication may also prove to be an important model for cellular responses mediated through the DNA-PK-related kinases, ATM and ATR.

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Induction of DNA Damage Responses by Adozelesin Is S Phase-specific and Dependent on Active Replication Forks[1]

Jen-Sing Liu,[2] Shu-Ru Kuo,[2] Terry A. Beerman, and Thomas Melendy[3]

Departments of Microbiology and Biochemistry and the Witebsky Center for Microbial Pathogenesis and Immunology, University at Buffalo, SUNY, School of Medicine and Biomedical Sciences, Buffalo, New York 14214 [J-S. L., S-R. K., T. M.], and Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York 14263 [T. A. B.]

Abstract

Adozelesin is an alkylating minor groove DNA binder that is capable of rapidly inhibiting DNA replication in treated cells through a *trans*-acting mechanism and preferentially arrests cells in S phase. It has been shown previously that in cells treated with adozelesin, replication protein A (RPA) activity is deficient, and the middle subunit of RPA is hyperphosphorylated. The adozelesin-induced RPA hyperphosphorylation can be blocked by the replicative DNA polymerase inhibitor, aphidicolin, suggesting that adozelesin-triggered cellular DNA damage responses require active DNA replication forks. These data imply that cellular DNA damage responses to adozelesin treatment are preferentially induced in S phase. Here, we show that RPA hyperphosphorylation, RPA intranuclear focalization, and γ-H2AX intranuclear focalization induced by adozelesin treatment are all dependent on DNA replication fork progression, and focalization is only induced in S phase cells. These findings are similar to those seen with the S phase-specific DNA-damaging agent, camptothecin. Conversely, all three DNA damage responses are independent of either S phase or replication fork progression when induced by treatment with the DNA strand scission agent, C-1027. Furthermore, we demonstrate that adozelesin-induced RPA and γ-H2AX intranuclear foci appear to colocalize within the nuclei of S phase cells.

Introduction

The anticancer agent adozelesin is an analogue of CC-1065, a CPI[3] isolated from *Streptomyces zelensis*. Adozelesin binds to the minor groove of A/T-rich DNA sequences and alkylates the N3 of adenines at the 3'-end of its binding sites (1, 2). This results in the formation of adozelesin:DNA adducts, which leads to the inhibition of both cellular and viral DNA replication and a S phase cell cycle arrest (3, 4). Previously published results show that the inhibition of DNA replication by adozelesin occurs through a *trans*-acting mechanism. The *trans*-acting replication factor that is inactivated on treatment with adozelesin has been identified as RPA (5). RPA is the major eukaryotic single-strand DNA binding protein. This heterotrimeric protein (M_r 70,000; 32,000; and 14,000) is essential for DNA replication and plays critical roles in DNA repair and recombination (6, 7).

The NH_2-terminal domain of the M_r 32,000 subunit of RPA (RPA32) has been shown to become hyperphosphorylated during S phase of the cell cycle. RPA32 hyperphosphorylation is also induced in response to DNA damage (6, 7). Shao *et al.* (8) reported that induction of RPA32 hyperphosphorylation by γ radiation or CPT could be prevented by pretreating cells with the replicative DNA polymerase inhibitor, aphidicolin. Similar results have also been observed in UV-irradiated and adozelesin-treated cells (5, 9, 10). These findings suggested that RPA32 hyperphosphorylation in response to DNA damage might be dependent on the active passage of DNA replication fork. However, when cells were treated with enediyne C-1027, the induction of RPA32 hyperphosphorylation was found to be completely resistant to aphidicolin (11). This suggests the presence of both replication-dependent and -independent mechanisms for RPA32 hyperphosphorylation.

CPT, a topoisomerase I inhibitor, is known to selectively induce S phase DNA damage checkpoints and causes DSBs only when there is DNA replication fork movement (12). This may explain why RPA32 hyperphosphorylation induced by CPT is dependent on replication fork progression. It is also understandable how C-1027, a DNA scission agent that directly binds and breaks one or both strands of DNA (11, 13, 14), can induce RPA32 hyperphosphorylation independent of replication fork movement. However, it is unclear why replication fork progression would be required for RPA32 hyperphosphorylation induced by agents like adozelesin, which directly damage DNA.

In S phase cells, a small portion of RPA becomes tightly associated with the nuclear matrix to form intranuclear foci, which were found to correspond with sites of DNA replication (15–18). RPA focalization was also observed in UV- or γ-irradiated cells, and these RPA foci colocalized with other
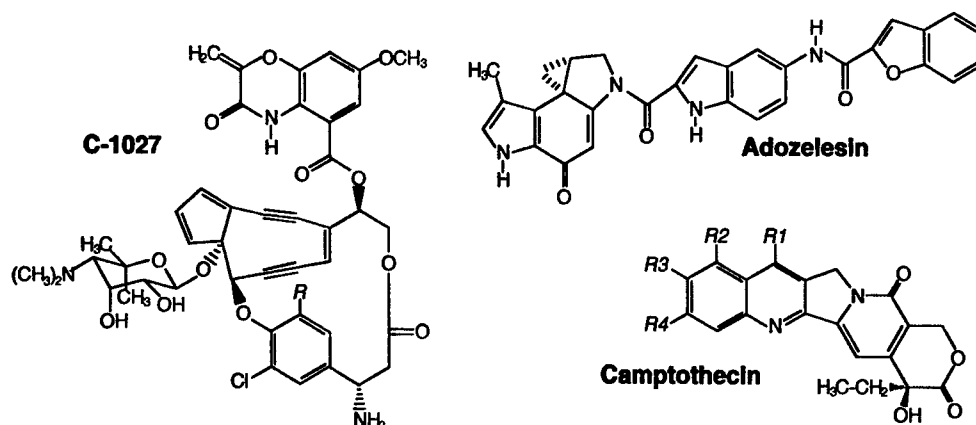
Fig. 1. Structures of DNA-damaging agents C-1027, adozelesin, and CPT.

DNA repair factors (19–23). It has not been demonstrated whether DNA damage-induced RPA focalization also has the same dependency on DNA replication fork progression as RPA32 hyperphosphorylation.

In this study, we study how treatment of human cells with these three DNA-damaging agents (CPT, C-1027, and adozelesin; Fig. 1) triggers these early cellular DNA damage responses and the role that S phase and DNA replication play in these responses. We have used relatively high levels of these three agents over short time frames to focus primarily on the early responses to DNA damage. Specifically, we addressed the question of whether adozelesin triggers these DNA damage responses in an S phase-specific manner. We examined phosphorylation of RPA and formation of RPA and γ-H2AX (histone H2AX phosphorylated at serine 139) foci in response to adozelesin, CPT, and C-1027. The induction of γ-H2AX foci is known as an early cellular response to either DSBs or replicational stress (for review, see Ref. 24). CPT and C-1027 were used as examples of S phase-specific and nonspecific DNA-damaging agents, respectively. Our results show that like CPT, adozelesin-induced RPA32 hyperphosphorylation and γ-H2AX and RPA focalization are S phase specific and require active DNA replication fork progression.

Materials and Methods

Chemicals and Antibodies. CPT (10 mM, dissolved in DMSO), aphidicolin (1 mg/ml, dissolved in ethanol), and DAPI were purchased from Sigma Chemical Co. (St. Louis, MO). Adozelesin, generously supplied by Pharmacia Upjohn Co. (Kalamazoo, MI), was dissolved in dimethylacetamide (2 mg/ml) and further diluted in DMSO before its addition into culture medium. C-1027, a gift from Taiho Pharmaceuticals Co. Ltd (Saitama, Japan), was diluted in water. Both BUdR and anti-BUdR monoclonal antibody were purchased from BD PharMingen. Monoclonal antibody against γ-H2AX was purchased from Upstate Biotechnology. The monoclonal antibody against RPA32 was described by Din *et al.* (25), and polyclonal antibody was raised against an MBP-RPA32 fusion protein (26) and affinity-purified against His-tagged

RPA32. Fluorescein-conjugated goat antimouse and Alexa 568-conjugated goat-antirabbit antibodies were purchased from Vector Laboratories, Inc. and Molecular Probes, respectively.

Cell Line and Cell Culture. Monolayer cultured HeLa cells (American Type Culture Collection) maintained in DMEM with 10% fetal bovine serum were grown in two-well chamber slides (Nalge Nunc International) for immuno-staining studies or in 60-mm plates for immunoblotting assays. Asynchronous cells were incubated with or without 2.5 μM aphidicolin for 1 h before and throughout the treatment. DNA-damaging agents were then added at the indicated concentrations. For S phase block, the cells were treated with 25 μM aphidicolin for 16–18 h. DNA-damaging agents were then added to the cell cultures directly or 1 h after changing to fresh medium. To monitor newly synthesized DNA, 20 mM BUdR were added to the culture medium for 30 min. After being replaced with fresh medium, these cells were then mock treated or treated with the indicated DNA-damaging agents.

Indirect Immunofluorescent Staining. The procedure for indirect immunofluorescent staining has been described previously (27). Briefly, after DNA-damaging treatments, cells were permeabilized and washed with 0.5% Triton X-100 in PBS to remove the free nucleosolic proteins, followed by paraformaldehyde (3% in PBS) fixation. The extraction-resistant RPA and γ-H2AX were stained with antigen-specific primary antibodies and fluorescein- or Alexa 568-conjugated secondary antibodies. DAPI (2 mM) was used to stain DNA. In experiments with pulse-labeled BUdR, a two-step staining protocol was used (17). Antigen-purified polyclonal RPA32 antibody and Alexa 568-conjugated goat-antirabbit antibody were first used to stain RPA. The bound antibodies were then fixed *in situ* with 3% paraformaldehyde in PBS at room temperature for 15 min. This was followed by acid denaturation of the DNA. BUdR-specific monoclonal antibody and fluorescein-conjugated antimouse antibody were then used to stain the newly synthesized DNA. RPA and γ-H2AX foci and BUdR incorporation were examined using an Olympus
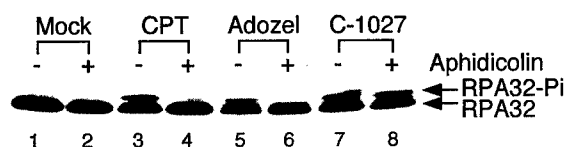
Fig. 2. DNA damage-induced RPA32 hyperphosphorylation in asynchronous cells. Exponentially growing HeLa cells were either mock treated (*Lanes 1* and *2*) or treated for 2 h with 50 μM CPT (*Lanes 3* and *4*), 40 nM adozelesin (*Adozel*; *Lanes 5* and *6*), or 1 nM C-1027 (*Lanes 7* and *8*), either with (even number lanes) or without (odd number lanes) pretreating the cells with 2.5 μM aphidicolin for 1 h (aphidicolin treatment maintained throughout DNA-damaging treatment). Total proteins were separated by SDS-PAGE and immunoblotted for RPA32. Migration of RPA32 and hyperphosphorylated RPA32 (*RPA32-Pi*) are indicated on the *right*.

Fig. 3. Formation of DNA damage-induced RPA and γ-H2AX foci in asynchronous cells. Monolayer cultured HeLa cells were either mock treated (*A*) or treated for 2 h with 50 μM CPT (*B*), 40 nM adozelesin (*C*), or 1 nM C-1027 (*D*), either with (*right panels*; *1 h Aphidicolin*) or without (*left panels*; *0 h Aphidicolin*) pretreating the cells with 2.5 μM aphidicolin, as in Fig. 2. Cells were then fixed and stained with DAPI, RPA32-specific polyclonal antibody, and monoclonal antibody against γ-H2AX as described in "Materials and Methods."

BX40 microscope with a SPOT-RT digital camera and software. Adobe PhotoShop was used for image processing and printing. The percentages of RPA-positive cells were calculated based on the number of DAPI-stained nuclei that were positive for fluorescein staining. For each preparation, the number was calculated using 200–300 cells and rounded to the nearest whole number.
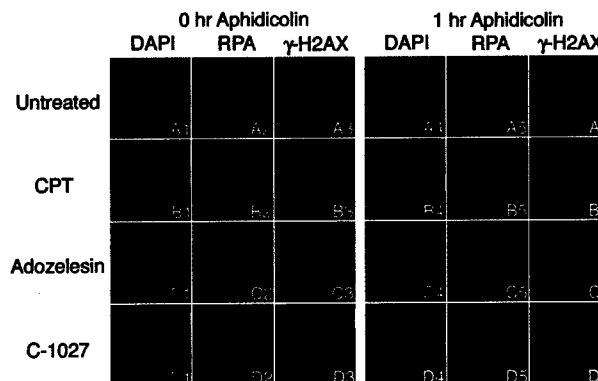
Western Blot Hybridization. Mock-treated or DNA-damaging agent-treated cells (1×10^6) were washed with cold PBS and lysed directly in SDS sample buffer [20 mM Tris-HCl (pH 7.5), 2% SDS, and 1 M 2-mercaptoethanol]. Total protein from an equal number of cells (~2×10^4) was resolved by electrophoresis on a 12.5% (w/v) SDS-PAGE and transferred to Hybond-P membrane using NovaBlot (Amersham Pharmacia Biotech) as per the manufacturer's instructions. The membranes were probed with a monoclonal antibody against RPA32 and peroxidase-conjugated goat antimouse IgG (Pierce). The membranes were then treated with Supersignal enhanced chemiluminescent reagent (Pierce) and exposed to X-ray film.

Results

DNA Damage-induced RPA and γ-H2AX Focus Formation. The monolayer cultured HeLa cells used in this study showed similar patterns of RPA32 hyperphosphorylation as human 293 cells from results published previously (5, 11). RPA32 hyperphosphorylation is triggered by treatment with as little as 1 nM adozelesin, 1 μM CPT, or 0.1 nM C-1027, with increasing levels of drug resulting in increasing levels of hyperphosphorylated RPA32 (Refs. 3 and 9 and data not shown). Aphidicolin pretreatment blocks RPA32 hyperphosphorylation induced by CPT or adozelesin at all levels tested (Fig. 2, *Lanes 3–6,* and data not shown) but not that induced by C-1027 (Fig. 2, *Lanes 7* and *8*). These results suggest that the activation of the kinase responsible for RPA32 hyperphosphorylation in cells treated with CPT or adozelesin requires DNA synthesis.

The effects of aphidicolin on DNA damage-induced RPA and γ-H2AX focus formation were then tested. Conditions for causing DNA damage in the presence or absence of aphidicolin were the same as that used in Fig. 2. After drug treatment, cells were washed with 0.5% Triton X-100 in PBS to permeabilize the nucleus and wash away the loosely associated nucleosolic proteins before fixation and immuno-

staining. A monoclonal antibody specific to γ-H2AX and polyclonal antibody against RPA32 were used as primary antibodies in indirect immunofluorescent staining. The nuclei of mock-treated cells contain very low levels of extraction-resistant RPA or γ-H2AX (Fig. 3*A1–A3*). Treatment of cells with any of the three DNA-damaging drugs induced high levels of intranuclear detergent-resistant RPA and γ-H2AX (Fig. 3, *B–D, Lanes 1–3*). However, the percentage of cells that contained RPA or γ-H2AX foci varied. Treatment of cells with CPT or adozelesin induced both RPA and γ-H2AX foci in ~38% of cells (Fig. 3, *B* and *C, Lanes 1–3*). In contrast, the vast majority of C-1027-treated cells (92%) contained high levels of RPA and γ-H2AX foci (Fig. 3*D, Lanes 1–3*). One hour of aphidicolin pretreatment eliminated the majority of RPA and γ-H2AX staining in cells treated with either CPT or adozelesin (Fig. 3, *B* and *C, Lanes 4–6*). In C-1027-treated cells, aphidicolin had virtually no effect on RPA or γ-H2AX focus formation (Fig. 3*D, Lanes 4–6*). These results demonstrate that DNA damage-induced RPA and γ-H2AX focalization, as well as RPA32 hyperphosphorylation, can be either sensitive (for CPT and adozelesin) or resistant (for C-1027) to aphidicolin.

Because RPA and γ-H2AX foci appeared in the same cells after treatment with either adozelesin or CPT, the possibility of colocalization of these two signals was examined. As shown in Fig. 4, under higher magnification, RPA and γ-H2AX foci show very similar patterns in cells treated with adozelesin. The merged image of the RPA and γ-H2AX foci shows these patterns to be highly similar, suggesting a high degree of colocalization.

Adozelesin-induced RPA Focus Formation in S Phase Cells. The ability of aphidicolin to block RPA focus formation and hyperphosphorylation in cells treated with adozelesin suggested that the adozelesin-triggered changes in RPA are S phase-specific responses. To address this possibility, asynchronous cells were pulse labeled with BUdR to identify cells in S phase. After several
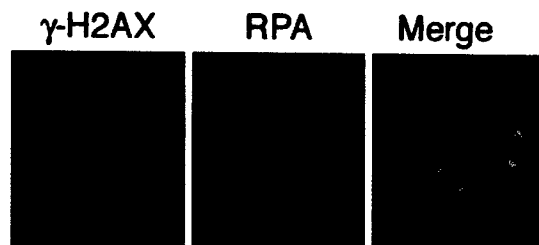
Fig. 4. RPA and γ-H2AX appear to colocalize in S phase nuclei in response to adozelesin treatment. An individual cell treated with 40 nM adozelesin as in Fig. 3C was analyzed using a ×100 objective lens. RPA staining is shown in *red*, and γ-H2AX staining is shown in *green*. Apparent colocalization is shown by the *yellow* signal in the merged image.
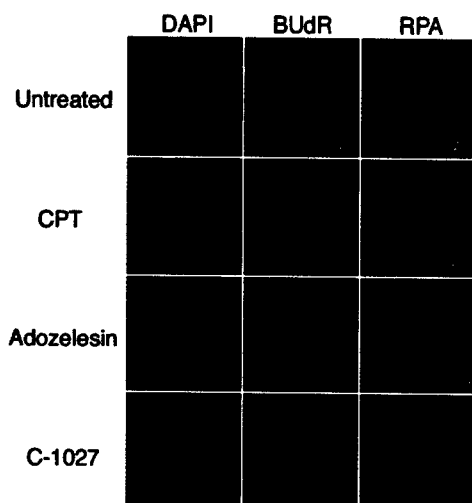

Fig. 5. DNA damage-induced RPA foci in asynchronous cells labeled with BUdR. Asynchronously growing HeLa cells pulse labeled with BUdR were treated with the 50 μM CPT, 40 nM adozelesin, or 1 nM C-1027 for 1 h. The total DNA is visualized by DAPI stain (*left panels*). Cells labeled with BUdR (stained *green*) shown in the *center panels* represent cells in S phase. RPA stained with polyclonal antibody against RPA32 (in *red*) is shown in the *right panels*.

Fig. 6. Formation of DNA damage-induced RPA foci in S phase cells. HeLa cells were arrested in S phase by treating with 25 μM aphidicolin overnight. The cells were then incubated with 50 μM CPT, 40 nM adozelesin, or 1 nM C-1027 for 2 h either directly (*left panels*; *Aphidicolin Arrested*) or after cells were released from aphidicolin block for 1 h (*right panels*; *Released*). Cells were fixed and stained for DNA (with DAPI) and RPA (with monoclonal antibody against RPA32) as described in "Materials and Methods."

washes with fresh medium, cells were either mock treated or treated with DNA-damaging agent for 1 h and then stained for both RPA and BUdR (see "Materials and Methods"). In the absence of DNA-damaging agents, ~35% of the cells show both BUdR incorporation and weak but clearly detectable levels of RPA focalization (Fig. 5A). This result suggests that those cells with weak RPA staining nuclei are in fact in S phase. As in Fig. 3, treatment with adozelesin or CPT results in strong RPA staining in a fraction (~35%) of asynchronous cells (Fig. 5, *B3* and *C3*). The cell nuclei that contain high levels of RPA foci show a nearly exact correlation to the nuclei that stain positive for BUdR (Fig. 5, *B* and *C*). As observed in Fig. 3, C-1027 induces RPA focus formation in virtually all cell nuclei, regardless of BUdR incorporation (Fig. 5D). These results suggest that adozelesin and CPT both trigger DNA damage-induced RPA foci only in S phase cells.

We then evaluated whether it is just S phase or actual replication fork progression that is required to induce RPA
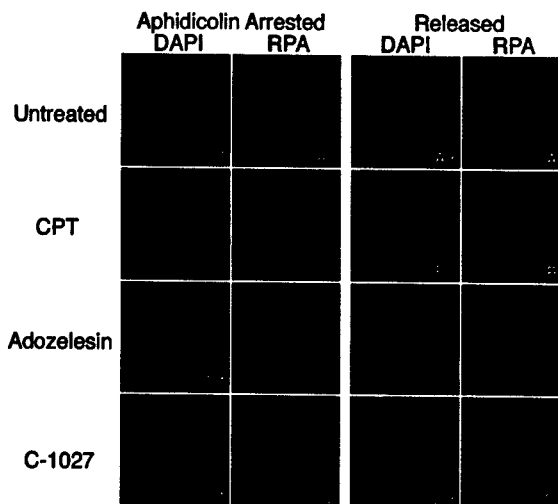
focalization. HeLa cells were enriched in S phase by incubating with aphidicolin for 16–18 h. As expected, these aphidicolin-arrested S phase cells (Fig. 6, *left panels*) showed no BUdR incorporation (data not shown). However, 1–2 h after their release from aphidicolin block, DNA replication activity resumed in these cells, and ~100% of the cells showed BUdR incorporation (data not shown). We defined these cells as released S phase cells (Fig. 6, *right panels*). Aphidicolin-arrested or -released S phase cells were then treated with DNA-damaging agents for another 2 h and stained for extraction-resistant RPA as described above. Treatment with CPT or adozelesin did not appreciably alter RPA staining in the aphidicolin-arrested cells (Fig. 6, compare panels *B2* and *C2* with *A2*); however, they induced high levels of extraction-resistant RPA foci in virtually all cells on release from aphidicolin block (Fig. 6, *B4* and *C4*). These results strongly suggest that CPT- and adozelesin-induced RPA foci are dependent not just on S phase but more specifically on replication fork progression. Treatment with C-1027 induced RPA focalization, regardless of S phase status or replication fork progression (Figs. 3D and 6D). It is noteworthy that although overnight aphidicolin treatment alone slightly enhanced RPA staining (compare Fig. 6A with Fig. 3A), even prolonged aphidicolin treatment did not induce appreciable RPA focalization.

DNA Damage-induced RPA32 Hyperphosphorylation in S Phase Cells. DNA damage-induced RPA32 hyperphosphorylation was also evaluated in aphidicolin-blocked or -released S phase cells. HeLa cells treated overnight with aphidicolin showed low levels of RPA32 phosphorylated to an intermediate mobility that were not appreciably changed after release from aphidicolin block (Fig. 7, *Lanes 1* and *2*).
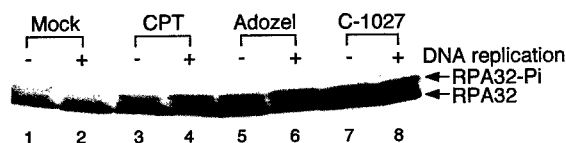
Fig. 7. DNA damage-induced RPA32 hyperphosphorylation in S phase cells. CPT (50 μM), 40 nM adozelesin, or 1 nM C-1027 were used to treat HeLa cells arrested with 25 μM aphidicolin for 2 h either directly (even numbered lanes) or after 1-h release from aphidicolin block (odd numbered lanes), as described in Fig. 6. Cells were lysed under reducing/denaturing conditions, and the lysates were separated by SDS-PAGE and immunoblotted for RPA32. Migration of RPA32 and hyperphosphorylated RPA32 are indicated on the *right*.

This likely represents the phosphorylation of RPA32 seen during S phase (6, 7). In several experiments, aphidicolin-arrested cells treated with either adozelesin or CPT showed either undetectable or very low levels of hyperphosphorylated RPA32 (Fig. 7, *Lanes 3, 5,* and *1* and data not shown). However, on release from aphidicolin block, which allows resumption of replication fork progression, >50% of the RPA32 in these cells became hyperphosphorylated (Fig. 7, *Lanes 4* and *6*). As with RPA focalization, C-1027 induced high levels of RPA32 hyperphosphorylation, regardless of S phase arrest or replication fork progression (Fig. 7, *Lanes 7* and *8*). These hyperphosphorylation results closely parallel those seen with RPA focalization (Fig. 6). Treatment with lower levels of these agents (as low as 1 nM adozelesin, 1 μM CPT, or 0.1 nM C-1027) resulted in decreased levels or intensity of γ-H2AX and RPA focalization and RPA32 hyperphosphorylation but showed the same cell cycle and replication fork dependence as seen with the higher levels of drugs, shown in the figures above (Refs. 3, 9, and 25 and data not shown).

Discussion

On the basis of the results presented above, in conjunction with reports published previously, we propose a model for how various types of DNA damage are affected by replication fork progression (Fig. 8). It is clear that for genotoxic agents that directly cleave DNA, such as C-1027, the broken DNA ends are capable of inducing various cellular DNA damage responses (Fig. 8A). Replication fork movement is therefore not required for the induction of either RPA or γ-H2AX focus formation or for the activation of the RPA32 kinase. These results show that DNA damage-induced RPA focus formation and hyperphosphorylation can happen outside S phase of the cell cycle, in contrast to the model suggested by others (8–10). Gamma radiation also creates DSBs; however, RPA32 hyperphosphorylation induced by γ radiation was largely blocked by treatment with aphidicolin (8). This may be explained in that DSBs only make up ~20% of the total DNA lesions in γ-irradiated cells (28). Some of these other lesions likely require DNA replication fork movement to induce cellular S phase checkpoint responses.

For indirect damage on DNA, such as CPT, or damage that is poorly recognized by cellular DNA repair pathways, such as DNA alkylation by adozelesin, the induction of RPA and γ-H2AX focalization and RPA32 hyperphosphorylation oc-
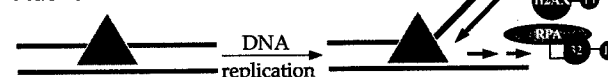
(A) Double Strand Break
C-1027
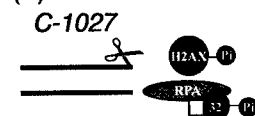
(B) Stalled Replication Fork + Double Strand Break
Camptothecin
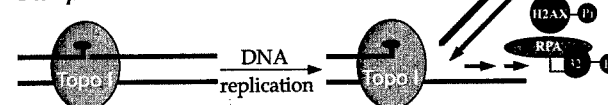
(C) Stalled Replication Fork + ?
Adozelesin

Fig. 8. Models for triggering S phase-specific DNA damage responses. Figure depicts three models for how different types of DNA lesions result in damage on replication fork passage: *A*, DSBs can be easily recognized without replication fork passage; *B*, CPT-induced topoI:DNA adducts only generate DSBs and RPA and γ-H2AX DNA damage responses on replication fork passage; *C*, similarly, poorly recognized DNA damage in cells that progress into S phase results in stalled replication forks that only induce RPA and γ-H2AX DNA damage responses on replication fork passage.

curs only in S phase cells with active replication fork progression. CPT and its derivatives are among the most frequently used anticancer drugs and have long been known to selectively induce S phase DNA damage checkpoints (for review, see Ref. 29). Specifically, CPT treatment can induce DSBs in treated cells only if DNA replication forks are in motion (Fig. 8B; Ref. 12). This likely explains the S phase specificity of these drugs. In this study, we show for the first time that like RPA32 hyperphosphorylation, CPT-induced RPA and γ-H2AX focalization also require replication fork progression.

In contrast to CPT, the alkylation of DNA by adozelesin *in vivo* apparently does not cause an appreciable number of DNA strand breaks (30) or distort the duplex structure of targeted DNA (2). There have been some indications that CPI-induced lesions may be removed by nucleotide excision repair (31, 32). However, adducts on DNA caused by the CPI drug CC-1065 were found to persist in CC-1065-treated BSC-1 green monkey cells (33). The absence of RPA and γ-H2AX foci in non-S phase cells suggests that adozelesin-induced DNA adducts are either virtually undetectable or intractable to DNA repair enzymes, until the collision of replication forks with the drug:DNA adducts results in stalled replication forks. Stalled replication forks have been shown to induce S phase checkpoint responses (34, 35). Because adozelesin treatment does not induce apparent DNA strand breaks (30, 33),[4] we conclude that adozelesin-induced, S phase-specific DNA damage and checkpoint responses must be triggered by stalled replication forks rather than DNA strand breaks. The S phase and replication dependence of

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[4] T. A. Beerman, unpublished data.

DNA damage responses triggered by adozelesin are of particular relevance because adozelesin treatment results in cells arrested predominantly in S phase (3, 4).

Phosphorylation on serine 139 of histone H2AX by ATM is an early cellular response to DSBs. Both C-1027 and CPT are capable of inducing DSBs in treated cells; therefore, γ-H2AX focus formation is expected. However, adozelesin-induced γ-H2AX foci would have to result from a different mechanism. A recent publication showed that γ-H2AX foci are formed in response to replicational stress induced by either hydroxyurea or UV treatment (36). The kinase responsible for this phosphorylation was shown to be ATM-Rad3-related protein kinase rather than ATM. Because adozelesin-induced γ-H2AX focus formation is dependent on replication fork progression, this pathway may also be dependent on ATM-Rad3-related protein kinase. Although aphidicolin is capable of inhibiting DNA polymerase activity and blocking replication fork progression, RPA and γ-H2AX focus formation and RPA32 hyperphosphorylation were not triggered to an appreciable extent by aphidicolin treatment alone (Figs. 3, 6, and 7). Hence, replicational stress alone is not sufficient to induce RPA or γ-H2AX foci. This may indicate that there is more than one type of checkpoint response to stalled replication forks, depending on the manner of blockage.

Acknowledgments

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